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An EBNA-1-Dependent Enhancer Acts from a Distance of 10 Kilobase Pairs To Increase Expression of the Epstein-Barr Virus LMP Gene

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Upon infection of human B lymphocytes, the 172-kbp Epstein-Barr virus genome forms a covalently closed circle via its terminal repeats. This event brings all of the promoters that control expression of the latent gene products, and the viral origin of plasmid replication, *oriP*, within a 20-kbp stretch of contiguous DNA. We have found that the EBNA-1-dependent transcriptional enhancer FR, located in *oriP*, increased the expression of a tagged viral oncogene encoding the latent membrane protein (LMP) up to 200-fold in normal Epstein-Barr virus-positive cells. The effect of FR was exerted across 10 kbp of viral DNA that spans the circularized ends of the viral genome. Enhancement of the tagged LMP gene by FR/EBNA-1 did not require the EBNA-2-responsive element.

Epstein-Barr virus (EBV) infects human B lymphocytes, causing the quiescent cells to become activated, to proliferate, and to yield cell lines efficiently, a process referred to as immortalization (reviewed in reference 13). In immortalized B lymphocytes, EBV is maintained in a latent state. Only a small subset of the viral genes is expressed, few or no virus particles are produced, and the plasmid viral genomes are replicated in synchrony with the cellular DNA.

One of the first recorded events following infection of a B lymphocyte by EBV is the circularization of the viral genome via its terminal repeats (10). As a result of circularization, all of the *cis*-acting elements that control expression of the latent genes are contained in 20 kbp of contiguous DNA that spans the covalently closed ends of the 172-kbp viral genome (Fig. 1). Ten viral genes are expressed during the initiation and maintenance of the latent infection (20). Several of these latent gene products themselves play roles in controlling the activity of the latent viral promoters.

The nuclear protein Epstein-Barr nuclear antigen 2 (EBNA-2), which is required for immortalization (3, 8), is the first detectable protein synthesized upon infection (1, 18). EBNA-2 appears to act as a switch that turns on expression from three latent promoters, Cp, LMPp, and Tp (4, 19, 23, 24). EBNA-2's activity at each of these three promoters is likely to be mediated by its association with the cellular protein RBP-Jκ, also termed CBF-1 (9, 12). Binding sites for RBP-Jκ are found within the regions defined as the EBNA-2-responsive elements upstream of each of the promoters (9, 11). Two of these EBNA-2-responsive promoters, LMPp and Cp, direct expression of the four other viral gene products that are required for immortalization.

Expression from Cp is also regulated by Epstein-Barr nuclear antigen 1 (EBNA-1) (21). EBNA-1's effect on Cp is mediated by the viral family of repeats (FR) located 3.5 kbp upstream of Cp. FR consists of 20 binding sites for EBNA-1 (15) and is part of the viral origin of plasmid replication, *oriP* (17). FR is required for viral replication and also functions as a transcriptional enhancer (16). Both activities of FR are dependent on the presence of EBNA-1. Knowing that FR/EBNA-1 can enhance the activity of Cp, we hypothesized that expression of the LMP gene would also be enhanced by FR/EBNA-1 following circularization of the viral genome. The known start site for transcription of the LMP gene has been mapped to position 169,516 (5), 10 kbp away from FR.

To test whether FR could enhance expression of the LMP gene, plasmid LMP(*oriP*$^+$), which contained a luciferase reporter inserted just upstream of the LMP translational start site, and a derivative of it that lacked *oriP*, LMP(*oriP*$^-$), were constructed (Fig. 2). These DNAs were engineered both to maintain the viral DNA between FR and the transcriptional start site for the LMP gene intact and to ensure that FR was at least 10 kbp distant from that start site in either direction around the plasmid DNA. Each plasmid was introduced into four EBV-positive, normal human lymphoblastoid cell lines, and the activity of the luciferase reporter gene was measured approximately 40 h after transfection. The results are shown in Table 1. In each of the four cell lines tested, expression of the LMP(*oriP*$^+$) reporter was significantly higher than that of the LMP(*oriP*$^-$) reporter, with the increase ranging from 30- to 200-fold. Thus, the presence of FR in its natural context within *oriP* of the EBV genome markedly increased expression of the luciferase reporter inserted into the LMP gene.

The two plasmids, LMP(*oriP*$^+$) and LMP(*oriP*$^-$), differ in that the former can replicate in EBV-positive cells while the latter cannot. To ensure that replication was not responsible for the increased activity of LMP(*oriP*$^+$) in comparison with that of LMP(*oriP*$^-$), a plasmid was constructed in which just the dyad symmetry (DS) element of *oriP* was deleted. The DS element is close to or is the site at which plasmid replication initiates (6) and is required for replication of *oriP* (17). The plasmid LMP(DS$^-$) (Fig. 2) retains FR but is not capable of replicating.

The activity of LMP(DS$^-$) was compared with that of LMP(*oriP*$^-$) 40 h after transfection into EBV-positive lymphoblastoid cell lines. The results are shown in Table 1 and demonstrate that FR, in the absence of plasmid replication, can enhance expression of the tagged LMP gene to levels similar to those achieved with intact *oriP*.

Having observed that the EBNA-1-dependent enhancer FR can dramatically increase expression of the tagged LMP gene

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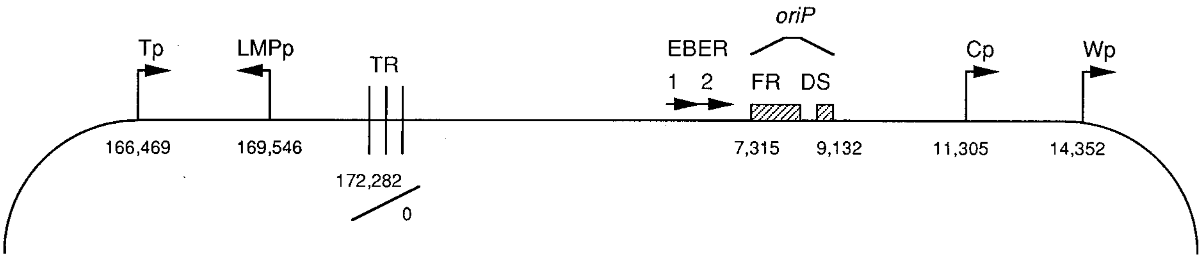

FIG. 1. All of the *cis*-acting elements that regulate the EBV genes expressed in latently infected B lymphocytes are contained within a 20-kbp stretch of the 172-kbp viral genome. This region spans the covalently closed ends of the genome (TR). Four latent promoters are indicated: Wp for EBNA-2 and EBNA-LP; Cp for EBNA-1, -3a, -3b, and -3c; LMPp for LMP; and Tp for the terminal protein (also termed LMP2). The genes encoding the two small RNAs EBER 1 and EBER 2 are shown, as is the origin of plasmid replication, *oriP*. *oriP* is composed of two elements, the dyad symmetry element (DS) and the family of repeats (FR). The numbers indicate genomic locations determined from the sequence reported by Baer et al. (2).

in normal EBV-positive cells, we sought to determine whether this activity was dependent on the presence of the previously identified EBNA-2-responsive element upstream of the LMP promoter (22). To test whether the EBNA-2-responsive element was required for the ability of FR/EBNA-1 to enhance expression of the tagged LMP gene, a 281-bp deletion was made in both plasmid LMP(*oriP*$^+$) and plasmid LMP(*oriP*$^-$). The deletion extended from positions −54 to −335 relative to the LMP transcriptional initiation site and included the EBNA-2-responsive element (Fig. 2). These plasmids were transfected into either GM2783 or RPMI 1788 cells, and the activity of the tagged LMP gene was measured. The results are shown in Table 2.

As expected, deletion of the EBNA-2-responsive element decreased the activity of the luciferase reporter inserted into the LMP gene in the two cell lines. Nonetheless, in the absence of the EBNA-2-responsive element the presence of FR resulted in an approximately 30- to 60-fold increase in activity of the luciferase reporter. Thus, the EBNA-2-responsive element is not required for this level of enhancement of the LMP gene by FR/EBNA-1.

The ability of FR/EBNA-1 to stimulate the LMP gene in the absence of other EBV gene products, particularly EBNA-2, was tested. The EBV-negative Burkitt's lymphoma-derived cell line BJAB was transfected with plasmid LMP(DS$^-$) in the presence or absence of an expression vector for EBNA-1. EBNA-1 stimulated activity of the luciferase reporter inserted into the LMP gene 10- to 25-fold in this cell line (data not shown). Thus, no other EBV gene products were necessary for this level of enhancement of the tagged LMP gene by FR/EBNA-1.

We tried to identify the initiation site for luciferase transcripts in GM2783 transfected with plasmid LMP(*oriP*$^+$). The expression of luciferase RNA was below the level of detection of S1 nuclease protection assays. The PCR-based technique rapid amplification of cDNA ends (RACE) was used to analyze both total RNA and poly(A)$^+$ RNA from GM2783 cells transfected with LMP(*oriP*$^+$). Following cDNA synthesis from

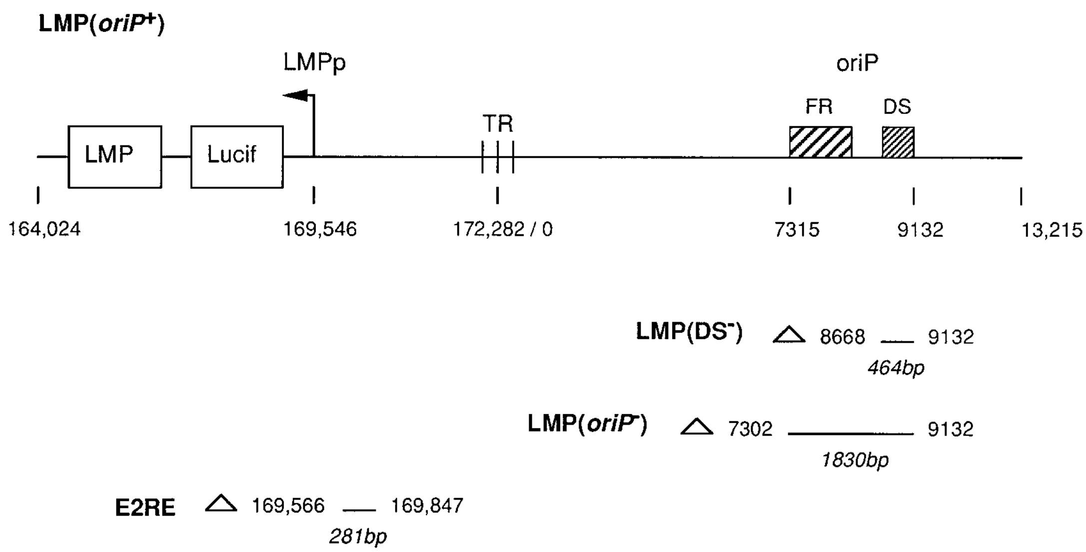

FIG. 2. Maps of the EBV sequences contained in luciferase reporter plasmids used to test the effect of FR on expression of the LMP gene. All numbers indicate EBV genomic locations and are determined from the sequence reported by Baer et al. (2). Plasmid LMP(*oriP*$^+$) contains 21.4 kbp of EBV DNA extending from the *Sfi*I site at position 164,024 of the EBV genome across the fused viral termini (TR) to the *Bam*HI site at position 13,215. The luciferase gene (Lucif) was inserted between the *Bsu*36I site at position 169,475 (+40 relative to the transcriptional initiation site for LMP) and the *Sfi*I site at position 169,441. The two components of *oriP*, FR and DS, are indicated. In the LMP(*oriP*$^+$) reporter, which is 26,783 bp in size, FR is approximately equidistant in the circular DNA from the transcriptional initiation site for LMP. LMP(DS$^-$) was made by deleting a 464-bp *Spe*I-*Hpa*I segment (EBV positions 8668 to 9132) from LMP(*oriP*$^+$). To make plasmid LMP(*oriP*$^-$), a 1,830-bp *Dra*I-*Hpa*I segment (EBV positions 7302 to 9132) was excised from LMP(*oriP*$^+$). The derivatives of LMP(*oriP*$^+$) and LMP(*oriP*$^-$) in which the EBNA-2-responsive element (E2RE) was deleted were made by excising a 281-bp *Mlu*I-*Bss*HII segment (EBV positions 169,566 to 169,847) which spans positions −54 to −335 relative to the transcriptional initiation site for LMP. These maps are not drawn to scale.

TABLE 1. EBNA-1-dependent enhancer FR increases expression of luciferase reporter inserted into LMP gene 30- to 200-fold in normal EBV-positive cells

Recipient cell[a]	Relative light units[b]				
	LMP($oriP^+$)	Fold increase[c]	LMP($oriP^-$)	Fold increase[d]	LMP(DS^-)
GM2783	141,000 ± 34,000	59	2,400 ± 200	46	111,000 ± 13,000
RPMI 1788	51,000 ± 10,500	204	250 ± 200	132	33,000 ± 3,800
11/17-5	14,000 ± 1,300	28	500 ± 350	32	16,000 ± 1,900
721	9,100 ± 2,900	114	80 ± 150	112	9,000 ± 1,800

[a] Cells were electroporated as previously described (7) with 1.5 μg of DNA per 10^6 cells. Cells were harvested approximately 40 h after transfection, and luciferase activity was measured in 2×10^6 cells.

[b] Each result represents the average for three independent experiments and is shown with its standard deviation. In each independent experiment, plasmids whose activities were being compared were all electroporated separately into a particular cell line. For each plasmid, three separate samples of cells were pooled immediately following electroporation. Variations due to differing transfection efficiencies between independent experiments are part of the standard deviation calculated for each result.

[c] The fold increase equals the activity of LMP($oriP^+$) divided by the activity of LMP($oriP^-$).

[d] The fold increase equals the activity of LMP(DS^-) divided by the activity of LMP($oriP^-$).

total RNA and two rounds of amplification with nested primers specific for luciferase, a single, very faint band of a size corresponding to initiation from the position equivalent to nucleotide position 169,516 of the EBV genome was observed after Southern blotting and hybridization with a probe specific for luciferase DNA. When poly$(A)^+$ RNA was used as the template for cDNA synthesis, a prominent band corresponding to initiation at position 169,516 was observed, along with several additional bands corresponding either to initiation sites within the 275-bp region surrounding position 169,516 or to priming artifacts of the RACE technique. No signals were detected from RNA isolated from nontransfected GM2783 cells.

These results are consistent with the notion that FR/EBNA-1 enhances initiation of transcription from the LMP promoter identified by Fennewald et al. (5). However, we cannot rule out the possibility that initiation producing luciferase transcripts is also occurring at other sites. Nonetheless, because the spacing, orientation, and nucleotide sequence from FR to the reporter gene in the plasmids are identical to those found in the viral genome, and because we have inserted the luciferase reporter into the LMP gene, we can conclude that the activity of FR/EBNA-1 in our assay is representative of FR/EBNA-1's activity in the context of viral infection. Therefore, these findings indicate that FR/EBNA-1 stimulates expression of the viral oncogene encoding LMP.

TABLE 2. EBNA-1-dependent enhancement by FR of expression of luciferase reporter inserted into LMP gene can occur in absence of EBNA-2-responsive element

Recipient cell[a]	Relative light units[b]				
	LMP($oriP^+$)	Fold decrease[c]	E2-RE del LMP($oriP^+$)	Fold increase[d]	E2-RE del LMP($oriP^-$)
GM2783	141,000 ± 34,000	3	53,000 ± 22,000	33	1,600 ± 900
RPMI 1788	51,000 ± 10,500	15	3,400 ± 800	57	60 ± 20

[a] Cells were electroporated as previously described (7) with 1.5 μg of DNA per 10^6 cells. Cells were harvested approximately 40 h after transfection, and luciferase activity was measured in 2×10^6 cells.

[b] Each result represents the average for three independent experiments and is shown with its standard deviation. In each independent experiment, plasmids whose activities were being compared were all electroporated separately into a particular cell line. For each plasmid, three separate samples of cells were pooled immediately following electroporation. Variations due to differing transfection efficiencies between independent experiments are part of the standard deviation calculated for each result.

[c] The fold decrease equals the activity of LMP($oriP^+$) divided by the activity of E2-RE del LMP($oriP^+$).

[d] The fold increase equals the activity of E2-RE del LMP($oriP^+$) divided by the activity of E2-RE del LMP($oriP^-$).

The knowledge that both EBNA-1 and EBNA-2 contribute to the regulation of expression of LMP is important for a full understanding of the mechanism of initiation and maintenance of a latent infection by EBV. The role of EBNA-1 may have particular relevance to the recently recognized association between EBV and some Hodgkin's lymphomas. In EBV-positive Hodgkin's lymphomas, expression of EBNA-1 and LMP, but not EBNA-2, is often observed (reviewed in reference 14). We conjecture that FR/EBNA-1 may enhance expression of LMP independently of EBNA-2 in Reed-Sternberg cells and thereby contribute to the viral gene expression that is characteristic of this disease.

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